Remarks

Applicant respectfully requests reconsideration of the present application, in view of the foregoing amendments and the reasons that follow.

A. Rejection under Judicially Created Doctrine of Obviousness-type Double Patenting

Claims 1-26 and 30-32 stand rejected as being unpatentable over claims 1-23 of U.S. Patent No. 6,221,848.

The enclosed Terminal Disclaimer obviates this rejection.

B..Rejection under 35 U.S.C. 112, Second Paragraph

The Office rejects claim 29 as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. In support of this rejection, the Office states:

> The phrase "the pharmaceutically acceptable vehicle is selected from the group consisting of a liposome, an adenovirus vector and a ligand-DNA conjugate" in claim 29 is vague and renders the claim indefinite...The pharmaceutically acceptable vehicle appears to be an inert material, such as a buffered saline, however, an adenovirus vector or a ligand-DNA is not an inert material. It is unclear as to the metes and bounds of what would be considered "pharmaceutically acceptable vehicle". If an adenovirus vector of a ligand-DNA is a pharmaceutically acceptable vehicle, then it is unclear whether the polynucleotide in claim 27 is within the adenovirus vector or ligand-DNA complex, or said polynucleotide is separated from said adenovirus vector or ligand-DNA complex.

Office action, pages 2-3. Applicants respectfully traverse this rejection.

As acknowledged by the Office, the specification states that "a pharmaceutically acceptable vehicle...can be used as a vehicle for administering a medicament because the material is inert *or* otherwise medically acceptable, as well as compatible with the active agent." Application, page 10, lines 6 and 7 (emphasis added). Aside from an uncorroborated statement that an adenovirus vector or ligand-DNA complex is not inert, the record is devoid of any

evidence or arguments that the phrase "the pharmaceutically acceptable vehicle is selected from the group consisting of a liposome, an adenovirus vector and a ligand-DNA conjugate" does not correspond in scope with that which applicant regards as his invention. The specification describes the characteristics of a pharmaceutically acceptable vehicle and the use of an adenovirus vector and a ligand-DNA conjugate as pharmaceutically acceptable vehicles. See, for example, the application at page 18, line 1 through page 20, line 2, and in Examples 1 to 4. In light of the application disclosure and the teachings of the art, the language of claim 29 would be clear to a person of ordinary skill in the art.

C. Rejection under 35 U.S.C. 103(a)

The Office also rejects claims 27-29 as being unpatentable over Hartman *et al.*(EP 0284105) in view of Gregory *et al.* US 5,670,488A). Applicants respectfully traverse this rejection on the basis that a *prima facie* case of obviousness has not been established.

To establish a *prima facie* case of obviousness, the Office must satisfy three requirements. First, the prior art relied upon, coupled with the knowledge generally available in the art at the time of the invention, must contain some suggestion or incentive that would have motivated the skilled artisan to modify a reference or to combine references. See *Karsten Mfg. Corp. v. Cleveland Gulf Co.*, 242 F.3d 1376, 1385, 58 U.S.P.Q.2d 1286, 1293 (Fed. Cir. 2001). Second, the proposed modification of the prior art must have had a reasonable expectation of success, determined from the vantage point of the skilled artisan at the time the invention was made. See *Amgen, Inc. v. Chugai Pharm. Co.*, 927 F.2d 1200, 1209, 18 U.S.P.Q.2d 1016, 1023 (Fed. Cir. 1991). Third, the prior art reference or combination of references must teach or suggest all the limitations of the claims. See *In re Wilson*, 424 F.2d 1382, 1385, 165 U.S.P.Q. 494, 496 (C.C.P.A. 1970). For the reasons discussed below, these requirements have not been met.

The Office cites Hartman *et al.* as disclosing a plasmid encoding human manganese superoxide dismutase (hMnSOD) in a buffer solution and a pharmaceutical composition

comprising the human manganese superoxide dismutase or its analog for treating a subject suffering from a disorder associated with the generation of oxygen free radicals. The Office further cites Gregory *et al.* as disclosing adenovirus vector Ad2/CFTR-1 or pseudo-adenovirus vectors (PAVs) as vehicles for delivering cystic fibrosis conductance regulator (CFTR) protein in conjunction with antioxidants, such as superoxide dismutase. See Office action, page 5.

The evidence and arguments of record fail to show how the art suggests the combination of the plasmid as taught by Hartman *et al.* with the adenovirus vector or PAVs as taught by Gregory *et al.*, or how a skilled artisan at the time of the invention would have expected that such combination to be operable. Even as late as the year 2000, the construction of recombinant adenoviral vectors was unpredictable and wrought with obstacles. See attached copy of Rusconi et al., "Vectors for Gene Delivery", *Gene Therapy for Rheumatoid Arthritis*, Ed., S. Gay, 2000, Humana Press.

The Office urges that a skilled artisan would have been motivated to combine the plasmid as taught by Hartman *et al.* with the adenovirus vector or PAVs as taught by Gregory *et al.* "in order to use the adenovirus vector as a gene therapy vehicle for treating cystic fibrosis in a subject as taught by Gregory with a reasonable expectation of success." Office action, page 6. However, the recognition of a need to treat cystic fibrosis does not render obvious the treatment that meets that need. "There is an important distinction between the general motivation to cure an uncured disease..., and the motivation to create a particular cure...Recognition of an unsolved problem does not render the solution obvious." *Cardiac Pacemakers, Inc. v. St. Jude Medical, Inc.*, --F.3d--, Nos. 02-532, -1559 (Fed. Cir. August 31, 2004).

Finally, the Office argues that "[i]t would have been obvious for one of ordinary skill at the time of the invention to substitute the plasmid or the human manganese superoxide dismutase as taught by Hartman with the adenovirus vector as taught by Gregory..." Office action, page 5. Even if such substitution were obvious, the resultant modification would still fall short of yielding the claimed invention as it would lack the requisite polynucleotide.

* * *

Based on the foregoing remarks, applicant respectfully requests withdrawal of all outstanding rejections. Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date 2 November 2004   By _____

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VECTORS FOR GENE DELIVERY

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Foreword

Gene therapy bases its rationale on the transfer of genetic components (genes or fragments thereof) into somatic cells, with the aim of preventing, correcting, or healing various types of disorders. After this introductory sentence you expect us to start telling you some of the marvellous achievements in setting up the tools that allow this transfer. However, before entering into the intricate details of vectorology please allow us the following clear-cut statement: "as of today there is no perfect or general vector for gene therapy and there won't be probably any in the foreseeable future". We hope that with this statement in mind, it will be easier for the readers to understand why there is still such a multitude of seemingly disparate efforts in establishing appropriate vehicles for the gene transfer.

Therefore, the efficacy of gene therapy largely depends on the properties of the chosen 'vector' for gene transfer and expression. The reader should be also reminded that there is still some ambiguity in the denomination 'vector' because this concept can be understood either as the mere cis-elements that compose the transferred sequence (that is the nucleic acid sequence arrangement) or as the vehicle/method which is utilised for the transfer of the required gene.

1. PROBLEMS INFLUENCING VECTOR'S CHOICE

1.1 Nucleic acids as medicine: megadaltons instead of kilo-daltons

In gene therapy the drug is a segment of either DNA or RNA and this imposes major constraints in the delivery. In conventional pharmacology the drugs are molecules of limited size (hundreds of Daltons) that either freely enter into cells due to their lipophilic character or are hydrophilic and destined to either act in the extracellular space or to be imported through specific biological channels. The classical pharmacological drugs are designed to act over a relatively short time and their therapeutic concentration is usually controlled by re-administration. A termination of the administration results in a dilution and termination of the pharmacological effects. Nucleic acids do not share many of the above properties: they have a large molecular size (1 megadalton for a segment of 1500 base pairs), are destined to work in the cell nucleus but are neither lipophilic, nor can count on a physiological import system. Once delivered into the nucleus they either integrate and persist for the rest of the cell's life or are maintained episomally for variable amounts of time. Therefore, the usual pharmacological strategies only marginally apply to the delivery of these monstrous molecules. For instance, in order to render them permeable to the cell membrane, one has to either compact them into lipid-con-

taining particles or into viral envelopes or capsids. This means that the units of delivery are no longer single, soluble molecules but relatively large (100-500 nm) and only partially soluble aggregates. This latter aspect makes the work with nucleic acids as medicines very arduous and still poorly reproducible in the complexity of a living organism. To conclude these considerations we will mention that in the jargon of the gene therapists, the transferred gene is also usually referred to as the 'transgene'. The use of this term will hopefully simplify the reading of the further paragraphs.

1.2. Correcting disorders derived from loss-of-function or gain-of-function

Genetic and acquired disorders result from an imbalance of metabolic functions, which are ultimately controlled by the genetic layout of the affected cells. The nature of the delivery vector and the properties of the transgene will largely depend on whether the therapy is aimed at inhibiting or supplementing (or enhancing) metabolic functions.

In most monogenic disorders, the phenotype is caused by a single loss-of-function that depending on the hyerarchical position of the affected gene can cause a simple or a very pleiotropic defect. For instance in cystic fibrosis or in muscle dystrophy, the lack of function of the corresponding genes results in rather circumscribed phenotypes. In this case the therapy shall be aimed at the organs that cause the most debilitating symptoms. Thus, it may be necessary to count on a rather well targeted delivery of the therapeutic supplemental gene. When trying to correct another monogenic disorder such as lack of factor VIII, the vector does not necessarily need to be targeted to the original tissue (the liver), since the corrective factor will be secreted virtually from any targeted tissue.

When trying in general to compensate loss-of-function disorders, the level of gene transfer will also not necessarily need to be 100% since in most cases a small percentage of cured cells will exert a corrective function (see also under

1.5). Therefore, there will be lower requirements in terms of efficiency of gene transfer on the chosen vector. For these reasons, monogenic disorders, though relatively rare and unattractive from the marketing point of view, have received a fair amount of attention by academically- or industrially-based gene therapists. On the other hand, when trying to control disorders derived from gain-of-function such as most hyper-proliferatory diseases (cancers, auto immune disorders etc) it will be more important to reach transfer levels close to 100% or to ensure at least that the transfected cells initiate a feed-back control on the still untransfected partners and produce a 'bystander effect' (1,2). Thus, the type of vector and the construction of the transgene have to be adapted to this task.

1.3. The choice of cis-elements: constitutive or regulated expression

The first level at which the properties of a vector are defined is the assembly of the regulatory and coding elements. In most of proof-of-principle experiments in which the transgene was a reporter gene, the promoter of choice was taken from the panel of conventionally strong constitutive champions (CMV, RSV or SV40). These promoters could be also considered for clinically valid therapeutic vectors, although their strength is strongly cell-specific. Therefore, in 'second generation' experiments we have witnessed the use of tissue-specific promoters, although with erratic results (3-6). In fact, the use of a genuinely tissue-specific control would circumvent the need of precisely targeting the delivery, since the regulation would be brought at the transcriptional level. However, our understanding of tissue specificity of transcription is restricted to relatively short cis-acting elements, whereas in chromosomal genes locus regulation occupies probably relatively extensive sequences (7,8). This is very relevant, since the currently available strategies that ensure long term expression are based on random integration in the host genome. This random integration is different in each individual transformed cell and leads to unpredictable

position effects that influence the expression of the transgene (4,9-11).

As learned from conventional transgenic animal models obtained by pronuclear microinjection (in which the transferred DNA also randomly integrates), in the majority of the cases the transgenes are silenced, and only a fully equipped locus is 'protected' from these erratic influence of the flanking regions.

Such large DNA segments are not compatible with the packaging capacity of most current vectors, therefore we are momentarily 'condemned' to use surrogate mini-regulatory elements (12,13).

For some disorders, another important goal is the search of bio-sensing cis-regulatory elements that can respond to metabolic status such as hypoxia (14,15), glucose levels (16), etc. These elements will be indispensable in the assembly of artificial glands that are designed to respond to natural balances of metabolites, thereby producing factors such as insulin or Epo in physiologically relevant and homeostatically controlled amounts.

Finally, an interesting collection of externally controllable cis-elements can be found in the literature, such as promoters that can be regulated by insect hormones (17), steroid antagonists (18-20), rapamycin or tetracycline derivatives (21,22). The advantage of these systems is that the action of the transgene can be pharmacologically regulated. These have been used with variable success in animal models, where the administration of the external drugs was shown to exert the anticipated effects on the gene expression (18). Four parameters are important in these vectors: a) the magnitude of control, b) the potential for immunogenicity of the regulatory factor, c) the cross-reaction of the controlling drug with resident metabolism, d) the half-life of the drug, the regulatory gene product and the target gene product which altogether determine the rapidity of the onset and fading of the response. The systems that use vertebrate regulators such as receptor mutants that respond to steroid antagonists have some advantage in their low immunogenicity, but have some disadvantage in potential cross-reaction with resident receptors. On the other hand, the immunogenicity of the popular tet-regulatory system, which has a high

magnitude of regulation but utilises a procaryotic regulatory factor, has not yet been fully assessed. This means that so far there is still a lot to optimise in this field and that no perfect system is yet available.

1.4. The three fundamental questions in gene delivery: efficiency, specificity and persistence

1 gram of tissue contains an average of 1 billion cells and the interstitial passages are rarely larger than 150-200 nm. These numbers should suffice to illustrate the first big problem in gene delivery: efficiency of transfer. To this we should add the non-specific binding of particles by the extra-cellular matrix and the consequent dilution of active principle. Finally, we should remember that there is no specific import for nucleic acids though the cell membrane and through the nuclear envelope (see 1.1 and 1.8). Therefore, it becomes evident that the best current vehicles are bio-particles that have naturally evolved the capacity to solve many of these problems: the viruses (see 1.8; 2.2 and 2.3).

Since it is not always possible to guarantee absolute specificity of gene expression (see above 1.3), we have sometimes to delegate the specificity to the delivery particle. To this aim several strategies have been designed (see 2.3.2 and 2.4.2 below).

Finally we should consider that when correcting chronic or progressively degenerative disorders, the transgene must persist and be active over a very long time, preferably for indefinite time. This is one of the most difficult tasks. Even if we manage to concoct the best regulatory regions that will prevent gene silencing through random integration in the genome (see 1.7), we cannot prevent the transformed cells to be lost by natural shedding such as in rapidly growing epithelia. This forces us to choose among two alternatives. a) target master stem cells that will be maintained throughout the renewal of the target tissue; b) accept the discomfort of periodical re-administration of the transgene. With partial exception of the bone marrow, and in spite of the spectacular re-

cent advances in stem cell research, we are not yet able to guarantee the efficient transformation of pluripotent precursors and so far we are forced to consider re-administration as inevitable for the long term correction of most chronic disorders. Re-administration brings with itself all the unpredictabilities of the immune reactions, specially but not exclusively when working with viral vectors (see below). Thus, we can affirm that at the state of the art, there is still no single or clear solution to the long term treatment of chronic conditions.

1.5. Efficiency of transfer and persistence of expression: not always 100% required

The former paragraph could lead a pessimist to the conclusion that chronic conditions will never be treatable by gene therapy. The good news come from the fact that for some conditions such as haemophilia (23) or cystic fibrosis and many others, a fraction of the natural levels of expression is sufficient to achieve therapeutic effects. When attempting to correct those conditions, it is sufficient to guarantee between 5 and 10% of transformation of the target tissue. Therefore, the corresponding vehicles do not need to sustain a 100% transfer, although the problem of persistence of the transformed cells is still relevant.

On the other hand, there are treatments that require neither high efficiency nor specificity nor persistence of the transfer. One example is DNA-based vaccination (24,25), where a permanent effect is achieved upon transient expression of a transgene. Another spectacular example is the corrections of critical limb ischemia (26) where the ectopic and transient expression of naked DNA injected intramuscularly brings about sufficient VEGF signal to rescue ischemic tissue. Analogous protocols are currently considered for treatment of other cardiovascular conditions where a short term treatment can produce long lasting beneficial effects. These examples should suffice to illustrate the concept that efficiency, specificity and persistence are not a major requirement in all cases of gene-assisted therapy, and this is of encouragement for all those who believe in this type of interventions.

1.6 Specificity: strategies, satisfactions and frustrations

The choice of the physical strategy for delivery determines the requirements to the vehicle. Gene transfer can be achieved *ex vivo* (for example in bone marrow explants) and in this case, the specificity and immediate immunogenicity (see below) of the vector are less relevant. In other protocols, specificity can also be achieved *in vivo* by local application (inhalation, double balloon catheter, intramuscular, intratumoral, brain stereotactic injection etc.). Also in this case the properties of the gene carrier are focussing on efficiency rather than on specificity, since this latter is defined by the administration protocol. Only in systemic delivery (intravenous injection) the problem of targeting becomes relevant. In the simplest cases, one can exploit the natural tendency of some organs such as liver and kidney to accumulate particulated drugs (27-30). However, these organs are not necessarily the targets in all disorders and this poses some serious problems of read-dressing the accumulation of the transgene-bearing vehicle (see targeting under 2.3.2 and 2.4.2). The problem is double, since, not only one has to devise specific docking elements on the carrier particles, but one has also to circumvent non-specific accumulation in the above mentioned organs (see one example in (31)).

1.7. Persistence and integration: to be or not to be? In either case you'll pay a fee

As commented above a reproducible and efficient method for inserting the transgene into a defined chromosomal location is still lacking. This situation causes two side-problems: gene silencing from position effects (see also 1.3) and random insertional mutagenesis (that will be further commented under 3.2). Therefore there is not yet a satisfactory protocol that ensures indefinite persistence of the transgene without causing the two

above-mentioned effects. This problem will be solved only when either locus-specific integration can be achieved (as originally hoped with the AAV vectors (32,33)) or when self-replicating and segregating artificial chromosomes (34) will be available. Until then, when we choose an integrating vector for our therapeutic protocol, we must be aware that we shall benefit of its potential to make the gene to persist but at the same time to randomly disturb resident functions and to be subject to uncontrollable position effects.

Of course we can choose to utilise a non-integrating vector such as an RNA virus, an Adenovirus (35) or an Herpes virus (36). In these cases, the transgene persists for a while but is not co-replicated when the host cell proliferates and is destined to be lost. Therefore the dilemma is in the choice of accepting the benefits of chromosomal integration and pay the fee of random silencing and insertional mutagenesis, or avoid these latter but paying the fee of non-permanent transformation.

1.8. Viral versus non-viral, who wins?

The non-viral modes of gene transfer include physical, chemical and biochemical protocols. Among the physical methods direct injection of naked DNA (37), pressure-mediated transfer (38,39), electrically enhanced transfer (40,41) and biolistic bombardment (42,43) have showed various degrees of efficiency. The chemicals/biochemical protocols include the use of cationic lipids and different compaction methods and each company or research lab claims to have the best results, although it is rare to see direct and extensive cross-comparisons in the published papers. Recently, the biochemical methods in which viral proteins are included to spike liposomes (33,44-46) have received increased attention, since they seem to promise enhanced gene transfer coupled to increased targeting. Although we can observe that in cell cultures DNA can be delivered to more than 99% of the cells, only a minor portion (3-10%) will ultimately transiently express the transgene (our unpublished results). This discrep-

ancy is due to the second barrier in gene transfer: the nuclear envelope (37,47). It is hypothesised that the majority of the transfected DNA is degraded in the cytoplasm and is not reaching the nucleus. Among the strategies that have been recently proposed to reinforce this second transfer we shall mention the attempts to link to the DNA oligopeptides containing nuclear localisation sequences (48,49, and references therein). In our laboratory we are exploring the possibility of using resident nuclear shuttles to favour the import of the transgenes into the target cells. The strategy has been named SMGD (Steroid Mediated Gene Delivery, Figure 1) and aims at using intracellular nuclear receptors as ferrying vectors for the transfected DNA. Nuclear receptors such as the steroid receptors have nanomolar affinities for specific ligands and are nucleophilic, therefore appear to be excellent candidates for efficient and specific shuttles for macromolecules that display et their surface the cognate ligands. To achieve this, we had to devise strategies to chemically 'decorate' the transgene with ligands. So far, we have obtained encouraging results with model compounds interacting with the glucocorticoid receptor (Ceppi et al. in preparation).

2. SMALL PARADE OF CURRENTLY POPULAR VECTORS

2.1 The simplest way: delivering naked or 'biochemically dressed' DNA

2.1.1 Structures and methods

Attempts to deliver naked DNA by direct intramuscular injection have been pioneered by J Wolff (47,50, and references therein). The initial encouraging results have prompted a series of emulatory protocols aimed at exploiting this simple delivery system for gene-based vaccination. The mechanism of DNA uptake by muscles is only tentatively explained (50) has so far precluded the rational design of improvements of the ef-

ficiency. In spite of its simplicity, this method has been the first ever bringing clinically relevant results in the treatment of critical limb ischemia with ectopic expression of VEGF (26).

Besides these straightforward but highly empirical approaches, a number of ways to enhance the uptake of DNA has been adapted from the long standing experience with cell-cultures. This has led to the reformulation of various combinations of liposomes, lipoplexes and poly-lipoplexes (51) and to other sophisticated receptor-ligand mediated internalisation systems (52). These efforts have built an important platform of technologies for general and specific gene transfer, although the efficiency of these transfer methods is still several logs inferior to the virally-based modes.

In general, to achieve anything between 0.1 and few percent of transfected cells, one has to employ a 10exp[4] to 10exp[5] molecules per cell, whereas with viral transfer 1 to 10 particles per cell can give up to 100% transfer. This poses also the problem of the kinetics of delivery. When added in one shot, the excess molecules are either lost, degraded or can generate non-specific immune reactions. Therefore, non viral transfer usually implies the continuous delivery over a period of time. Recent advances in the design of bio-degradable microspheres or encapsulating biol-polymers that progressively liberate trapped DNA has shed new perspectives on this strategy (28,53-56). Still, when working with non-viral delivery, one has to deal with the meager efficiency of nuclear transfer of the transfected DNA (see above). This problem subsists independently to the delivery method and is one of the reasons of the large ratio (active molecules per cell) that is required to achieve reasonable transformation rates. Finally, there is no specific mechanism for integration into the host genome, and this relegates non-viral transfer to the realm of transient treatments, unless some strategy to improve integration or persistence of the transgene can be established. For these reasons, transfer of naked DNA is currently only indicated for DNA-based vaccinations, which nevertheless held a phenomenal potential in the prevention of infectious diseases and cancer (25).

2.1.2 Targeting

The use of microsphere-aided delivery (see above) can help in specific augmentation of local concentration of the active molecules, thus providing a sort of topical specificity. Other ways of simple targeting are offered by the body anatomy which permits molecular treatment of mucosae, epithelia etc. When injected systemically, each liposome formulation displays minor differences in the preferences of organ accumulation (28,30). However, the common tendency is that formulations accumulate in liver and kidney and are not able to pass blood-brain barrier (36,57). This natural condition will be an important hurdle in designing specifically targeted formulations. Drastic protocols such as liver by-pass (31) are good for a proof-of-principle of how to short-circuit this problem but are too laborious and invasive to be clinically implementable in a generalised manner.

A major effort has also been devoted to the identification of molecular components of the endothelial zip-code system (58,59). Once understood, this tissue-specific marking of the vascular system could provide an elegant system of local accumulation of active particles.

At the cellular level, the best candidates protocols in particle targeting are the ones that exploit affinity of ligands for surface receptors (52). Some important improvements have been achieved by preparing 'virosomes' (33,44) in which viral proteins are decorating the surface of liposomes. If proven to be reproducibly infectious and compatible with large scale preparation, these hybrid particles can pave the way to the generation of "artificial viruses" that may enjoy the advantages of in vitro assembly and avoid some of the disadvantages of biologically assembled viruses (commented under 2.5.3).

2.1.3 Advantages and disadvantages

The production of in vitro assembled particles can be better controlled and guaranteed be devoid of adventitious infectious pathogens. Therefore these for-

mulations are pharmacologically safer than biologically assembled infectious particles. Secondly, there will be less constraints on the size of the transgene, which is a major problem in biologically assembled viral particles (see 2.3). Furthermore, the composition of synthetic particles can be designed to be devoid of immunogenic elements. Therefore, in vitro formulations containing nucleic acids can be considered suitable for multiple re-administrations, a property that is not yet guaranteed with the most popular viral vectors. The lack of immunogenicity should however not be overemphasised, because DNA that has been conventionally amplified in bacterial systems (the most convenient basis of molecular large scale preparation) acquires some intrinsic immunogenicity because of the loss of methylation of CpG -rich sequences (60-63) and perhaps also because of some other newly acquired bacterial methylation patterns (62, our unpublished observations). Those problems can be theoretically solved, although there is no clear picture of the mid- and long-term reaction of an immune-competent organism subjected to repeated DNA delivery that does not go through the digestive tract.

The major disadvantage of non viral transfer methods remains obviously the intrinsic low efficiency of transfer. The improvements of the last five-ten years have increased the transfer rate perhaps by a factor of ten, but there are several logs to catch up with the viral transfer (see 2.1.1). If no breakthrough comes into the field, one has to assume that non-viral gene transfer will be restricted to the treatment of conditions where efficiency of the transfer is not a crucial parameter.

2.1.4 Suitability and examples

Paradoxically to its intrinsic inefficiency, non-ßviral gene delivery has been the first treatment to demonstrate unequivocal therapeutic value. This occurred in the seminal experiments by the research team of Jeff Isner (26), in which the expression of a pro-vascularising factor (VEGF) through simple intramuscular injection of a suitable recombinant plasmid has rescued necrotising limbs al-

ready in phase I trials. We are looking forward to confirmations of these encouraging data in phase II and phase II trials, as well as their extension of other treatments in the cardiovascular field. The trials of Victor Dzau and colleagues (39) are another success story in non viral gene-assisted treatment. Artery by-pass based on vein grafts currently fails in a high proportion due to the aberrant growth of the intima, induced by the higher blood pressure in the transplanted vein. The team of Dzau has pre-treated the graft with a simple pressure-mediated gene transfer and shown that the transferred genes were able to inhibit the hyper-proliferation of the smooth muscle cells, thus reducing significantly the occlusion of the graft. The transgenes chosen were either growth-inhibitory genes or decoy-oligonucleotides that transiently titrate transcriptional factors that are essential for the expression of pro-proliferatory genes.

We conclude this paragraph by mentioning the discovery of the interesting properties of the herpes viral protein VP22 (64). This protein is capable of cell-to-cell transfer and can translocate into cells even when added to the extracellular medium. This property extends to some VP22 chimeric proteins (65, and references therein). This may be just the tip of the iceberg of several new protein-mediated macro-molecular transfer systems that may solve some of the current problems of non-viral transfer, provided they can be designed to be invisible to the immune system.

2.2. Replication-defective and replication-competent viruses

2.2.1 General features of replication-defective recombinant viruses.

Viruses have evolved since millions of years to become professional gene-porters. They can exploit the most sophisticated molecular mechanisms to escape immune surveillance, to specifically dock to target tissues, to enter through cell membranes, to resist intracellular degradative enzymes, to deliver nucleic acids to the nucleus or to organise specialised compartments for genome repli-

cation and expression, to integrate into the host genome or to remain latent for several years in host organisms and to be even passaged through the germ line. Therefore, there is no better vehicle that can be envisioned by gene therapists to efficiently transfer the preferred therapeutic gene. Viruses have however a very nasty property: they are pathogenic because their capsid proteins are mostly immunogenic or toxic and their genetic reprogramming strongly disturbs the cell metabolism and cause diseases of various severity. A better knowledge of the viral genomes has permitted the distinction of segments necessary for the packaging into the capsid from those encoding replicative functions and capsid or envelope components. This has permitted to construct viruses that retain only fragments of their genome and are debilitated in some pathogenic functions. These defective viruses need to be amplified either in specialised packaging cells or in presence of helper viruses that provide *in trans* the missing functions. The situation is different for each virus and it would be too intricate to comment all the sequence geographies, thus I will take the example of the adenovirus to illustrate the steps undertaken to optimise transfer vectors for gene therapy. The adenoviral genome consists of 36 kb of linear DNA with inverted terminal repeats that are indispensable for replication and packaging into the capsid (35,66). The 'left' portion (E1/E2 in Figure 2A) contains the early genes whose expression is indispensable to prepare the conditions for genome replication (66). Other early functions are scattered in other regions and are dispensable for replication. The remaining 80% of the genome is occupied by the late genes, mostly expressed through the major late promoter (MLP, Figure 2A) and giving rise to variegated proteins through differential splicing. In packaging cells, the 'early' portion of the genome could be anchored into the chromosomes and shown to be functioning in trans. This permits the growth and assembly of viral genomes whose early segment is either missing or substituted by a transgene of interest (figure 2B). This scheme has been maintained in all the viruses of so called first and second generation. Those recombinant viruses

have proven invaluable to demonstrate efficient gene transfer in animal models and also in patients (33,66-68). However, the remaining leaky segment encoding late genes confers a significant immunogenic potential to those generation I vectors. Therefore, the expression in immune competent animals is restricted to few weeks. Several strategies have been proposed to reduce this immunogenicity (66). The best solution so far has been offered by the so-called 'gutless' (also called 'helper dependent' or 'high-capacity' or 'third generation') adeno-vectors. In these constructs the entire late region is replaced by a 'neutral' DNA segment including the therapeutic gene(s) (Figure 2C) and the recombinant genome is grown and packaged in presence of a helper virus whose assembly is repressed by various strategies (66,67). After careful purification one can obtain significant titers of the recombinant vectors that are minimally contaminated (approximately 10exp[-4]) by the helper virus. Extremely encouraging results have been recently reported with these gutted Adenovectors that were shown to produce a permanent somatic gene alteration that can persist for several months (69,70) even in immunocompetent animals. We are confident that clinical trials involving gutted viruses will confirm the compatibility of these vectors with long term correction by gene transfer.

2.2.2 Principle of replication-competent viruses

Several viruses encode early proteins that interact with ubiquitous tumor suppressors such as P53 or retinoblastoma. By exploiting this situation, some research teams have developed recombinant viruses that maintain a conditional replicative potential whose fulfilment depends on the absence of tumor-suppressor functions. Selective or at least preferential replication has been reported for adenoviruses that retain the E1B gene (71-73), for HSV (74-77) or other RNA viruses (33). These viruses are capable of lithic growth in cells that are missing or underexpressing tumor suppressors and this property makes them attractive candidates for tumor treatments, leading

to some interesting clinical protocols. Only the future will tell whether these expectations are well placed and whether these oncolytic viruses can be safely used either as stand-alone or as combination-treatment in tumor therapy.

2.3. bio-weapon 1: DNA viruses

2.3.1 Structures and methods

In the preceding paragraphs we have illustrated the principles behind recombinant Adenoviruses. Therefore we will not comment further on these developments. With analogous protocols two other important DNA viral carriers have been designed: the adeno-associated viruses (AAV) and the herpes viruses (HSV). These two are distinguished by diametrically opposite properties. The recombinant AAV particles have a very limited packaging capacity (3.5-4 kb) and integrate the recombinant DNA into the host genome (32,36,78). The intact AAV has the capacity of integrating specifically into a site on chromosome 19 but this property is missing in the emptied recombinant genomes, which integrate randomly. In spite of this random integration, the recombinant-AAV constructs seem to be refractory to gene silencing through position effects. this property is currently attributed to the AAV terminal repeats that seem to possess some kind of 'insulator' property that renders the intervening sequence rather independent of the integration context (79). The increasingly simple protocols for production of r-AAVs and the extremely low immunogenicity and toxicity of these particles has prompted a large number of investigations of therapeutic gene transfer into small and large animals (13,80-83). Therefore, these vectors promise to be a reasonable choice for the permanent transfer of small-sized constructs. The construction of HSV vectors is more laborious but offers several advantages. Particularly interesting is the possibility of generating recombinant genomes of very high capacity (up to 150 kb (33,36,84)). This opportunity could permit the transfer of large loci or of multiple regulatory cas-settes for precise tuning of gene expression.

Recently, an interesting variation on the theme has emerged: the possibility of combining the advantages of two independent viruses. So for instance hybrid genomes that combine the great infectious capacity of Adenovectors and the possibility of integrating the transgene through a surrogate retroviral transposition have been proposed (32,33,73,78). Analogously, hybrid HSV-AAV vectors that combine the large capacity of packaging of HSV and the integration power of AAV have also been proposed (32,33,36,84). These hybrid viral vectors are probably still at their rudimentary stage, but represent exciting developments in a field that would otherwise have stagnated over the intrinsic limitations of each individual carrier.

2.3.2 Targeting /retargeting

Every virus has a natural tropism which is defined by the host molecular partners required for infection, replication and packaging. When working with recombinant viruses the primary factor is represented by the infection mechanism. This is dependent on the match between the cell surface receptors / co-receptors and the docking sites displayed at the surface of the infectious particle. There are essentially two ways of changing the infectivity tropism: one is to alter the docking proteins (for instance the adeno fibres (68,73,85,86)) by adding protein domains that represent ligands for alternative receptors; another consist in preparing Janus-type ligands that on one side interact with the original docking structure and on the other face they provide a new ligand (85). The latter strategy has the advantage of maintaining the same general structure of the vector and is probably preferable to the former strategy which can alter the properties of the capsid and requires specially engineered packaging cells for the amplification of the recombinant vector. Both strategies have been tried in cellular and animal models, but the success has been so far only moderate, since the new specificity imposed a high price in infectivity. These approaches will require

substantial improvement before being suitable for serious clinical trials.

2.3.3. Advantages / disadvantages

The recombinant DNA viruses mentioned above share several advantages. They can be grown at very high titers (between 10exp[9] to 10exp[11] per ml), they have a very stable genome and they can efficiently infect both proliferating and non proliferating cells. This latter property is certainly the most attractive for somatic gene transfer, since many therapies would require the gene transfer in cells that do not proliferate such as neurons, endothelial cells etc. Adeno and HSV do not integrate their genome, and this can be considered as both advantageous and disadvantageous. The persistence as unintegrated genomes makes them immune to position effects (good news) but also implies the dilution of the recombinant genome upon proliferation of the infected cells (bad news). The recombinant AAVs combine the capacity of integrating in the host genome and a partial resistance towards position effects through their inverted terminal repeats (ITRs). However, a major disadvantage of AAV is the restricted packaging capacity (at most 4 kb of foreign DNA) while r-Adenos of the first generation can carry transgenes around 8 kb and 'gutted' adeno can accommodate more than 30 kb or HSV episomes can arrange up to 150 kb (36,84). The immunogenic potential of the DNA viruses is different fore each vector, with r-Adenos of the first generation being the most immunogenic and thus suitable only for unique treatments and for treatment of disorders where a certain immune reaction may even be advantageous such as cancer.

2.3.4. Suitability and examples

The literature on pre-clinical studies with DNA viral vectors is extremely wide and covers all possible disease models from cancer to infectious disorders. However, only very few reports bring convincing evidence of potential up-scalability to large animals. For several years, hyper-critical circles used to say that gene therapy has until now "only been good to cure mice". In spite of these sarcastic

affirmations, progress in some areas has been rather spectacular. The natural tendency of adenoviruses to accumulate in the liver after systemic administration had prompted a series of interesting trials for the correction of metabolic disorders (33,66,73). Unfortunately, the tragic events linked with one of these trials (87-91) has slowed down the experimentation with further improved Adenovectors, including gutted adenos. On the other side, the transduction of recombinant AAV expressing blood clotting factors has been demonstrated to provide long term therapeutic benefits in both small and large animal models (33,80,82,83,92). Similarly, the use of HSV and gutted Adenoviruses in pre-clinical tests has shown convincing persistence of gene expression in many tissues (33,82). Thus, DNA-viruses remain powerful tools for the treatment of both acute and chronic conditions.

2.4. bio-weapon 2: RNA viruses

2.4.1. Structures and methods

RNA viruses tend to have a relatively small and less stable genome compared to DNA viruses. The unstability of RNA genomes is mainly due to the lack of proofreading in RNA replication and this results in error rates in the range of 1 mistake in 10'000 nucleotides. Most RNA viruses can only transiently persist within a cell, but the retroviruses can convert their RNA genome into a cDNA which is transposed into the host genome and thus can be virtually carried indefinitely within the host cell. This latter property has immediately evidenced retroviruses as preferred vectors for gene transfer. The inspiration of the ability of retroviruses to carry foreign genes certainly comes from the early discovery of murine and avian oncogenic retroviruses which can transduce cellular protooncogenes. The first attempts to construct engineered retroviruses was indeed based on murine retroviruses. The essential cis-elements that must be carried along with the engineered genome are the long terminal repeats (LTR), the packaging region and the

primer annealing sequence (93). The genes for the necessary proteins (reverse transcriptase, capsid and envelope protein) can be transferred to host chromosomes in packaging cells. Once transfected with a plasmid encoding the engineered retroviral backbone, these packaging cells can produce infectious particles at titers around 10exp[6] 10exp[7] (93). The engineering of recombinant lentiviruses is more laborious since several regulatory proteins must be concertedly expressed to allow packaging (33,36,94). Currently the best system seems to be via co-transfection of trans-complementing plasmids (95). The complexity of the procedure is such that it cannot fully exclude the arising of recombinant genomes that are capable of autonomous replication (RCP, replication competent particles). The latent chance of the emergence of these RCPs has certainly strongly hampered the clinical implementation of protocols with lentiviral backbones, and this in spite of the obvious advantage of lentivirus over other retroviruses: their capacity of infecting quiescent cells (94).

2.4.2. Targeting /retargeting

The viral envelope (consisting of host cell membrane spiked by env proteins) can be virtually substituted or engineered at will. The currently used winning horse is the VSV env protein (93). Several attempts to engineer the env proteins to change their docking specificities have been reported. These attempts have invariably resulted in lower titers since there is little way to rationally redesign docking surfaces. Given the improvements in recombinant virus handling we can anticipate that new specificities will be obtained through genetic selection from combinatorial libraries rather than through rational design. Alternatively, changes of tropism can be obtained as for the Adenovirus, by coating a general env-protein with bifunctional ligands that on one hand mask the natural specificity and on the other hand contact alternative docking sites (see also 2.3.2). Unfortunately, it is not guaranteed that all docking sites will be suitable for viral internalisation, which is a rather complicated process.

2.4.3. Advantages / disadvantages

The major advantage of retroviral backbones is that they can be assembled free of viral-protein coding genes. This means that after integration a properly engineered provirus will not encode for immunogenic viral proteins, thus guaranteeing a long term survival of the transduced cell. The major advantage of lentiviruses is the capacity of infecting non-proliferating cells (see also 2.4.1), a situation which is advantageous for the treatment of differentiated tissues or slowly turning-over tissues such as the CNS, endothelium, or bones.

A major disadvantage of retroviruses is the low titer (two to four logs lower than the one of DNA viruses. A standard in vivo therapy may require between 10exp[12] an 10exp[13] infectious particles. For DNA vectors this means processing volumes of less than 1 litre, whereas tens to hundreds of litres may be necessary to obtain the same amount of retroviruses. This situation may become a strong drawback for the industrial preparation of clinical materials. The more subtle disadvantage of retroviruses is the intrinsic low-fidelity of replication. Assuming 1 mutation for every 10 kb, every third copy of a 3 kb transgene sequence will contain at least one mismatch. This can result to the loss of the biological activity in a large fraction of the transduced cells. Finally, as with AAV systems, the random integration into the host genome and the rather limited (about 9 kb) packaging capacity pose problems of long-term maintenance of gene expression (see above 1.7) and of insertional mutagenesis.

2.4.4. Suitability and examples

The very first bona fide gene therapy clinical trial (treatment of ADA deficiency (96)) was indeed performed with recombinant murine retroviruses. Since then retroviruses have been used to carry compensatory genes or toxic genes in many clinical protocols. However none of these attempts could demonstrate significant therapeutic effects, specially because the experiments were confined to phase I status, where the major focus is the measurement of side-effects. From

this point of view the retroviruses have at least confirmed their relatively low toxicity. The major draw-back of recombinant murine retrovirus is their inability to infect quiescent cells, but this property is rescued in the lentiviral systems (see 2.4.1), which have shown to be equally versatile and yet capable of infecting post-mitotic cells (94). The improvements in assembly will soon permit to produce recombinant lentiviruses guaranteed free of replication-competent particles, and this will open their option for clinical trials.

Finally, the simplicity of the original murine retroviral system already used in the very first (and essentially failed) gene therapy attempts by Anderson or Bordignon (96,97), has not discouraged the team of Alain Fischer who ultimately has brought the very first historical example of permanent radical cure of a genetic disorder by the ex-vivo transduction of bone marrow transplants with vectors supplementing the IL2-receptor gene which is defective in a rare form of SCID (82,98). The two young patients of Dr. Fischer are the best results that gene therapy has scored within its short adventure, and for the public opinion are more worth than thousands of pre-clinical 'mouse therapies' or reporter assays. Resting on these encouraging indications, gene therapy based on simple viral vectorology may indeed pave the way to several efficacious intermediate clinical applications. Whether or not this strategy will persist in the long term, it depends only on further improvements, but we can say that, if wisely used, the today's vectors already have a significant potential for the treatment of chronic disorders.

2.5. Macromolecular weapons: a short click at the *www.fantasticoligo.com* site

2.5.1. Structures and methods

Gene therapy does not require the transfer of full genetic complements to become effective. Small gene fragments, in form of synthetic single stranded or double stranded oligonucleotides can exert a powerful control on gene expres-

sion. The most popular form of oligonucleotide-assisted therapy is done with antisense sequences that are destined to block either the maturation or the translation of specific mRNAs. Protocols aimed at tumor control through oligonucleotides that down regulate the production of protoonogenic proteins or antiapoptotic factors have achieved the phase III (99). A further sophistication is the use of oligonucleotides that have a ribozyme function (100) and are able to specifically hydrolyse target mRNAs. Also double stranded 'decoy' oligonucleotides that compete with genomic sequences by binding transcription factors have been successfully used in the clinic (39).

A further level of sophistication is represented by oligonucleotides that are capable of forming triple-helix structures with specific target sequences. For triplex formation, the only a-priori requirement for the target sequence is that purines and pyrimidines should be segregated on the two strands (101, and references therein). Oligonucleotides capable of repressing or stimulating gene expression have been designed in this manner (102,103). Recently, triplex forming oligos capable of guiding molecules that induce specific repair of target sequences have been proposed (104). The major disadvantage of oligonucleotides is their short survival within a cell. Therefore, these protocols imply either continuous supply of the therapeutic oligo or single treatment of acute conditions. Several modifications of the desoxy-ribonucleotide backbone have been proposed to augment the resistance of oligos toward degradative nucleases. The most spectacular modification is probably the so called PNA (protein-nucleic acid) backbone, in which the carrier polymer no longer a phosphate-ribose chain (105,106, and references therein). A great advantage of PNA is certainly the lower negative charge of the polymer chain, which enormously stabilises either the double or the triple stranded structures that it undergoes with target sequences. PNA has been shown to be easy to handle and to permit also accumulation into cellular subcompartments such as mitochondria (107), a condition which is not met by other nucleic acid transfer methods. We are certain that

PNA will become the polymer of choice for the locus-specific accumulation of active principles.

Finally, a bizarre family of chimeric oligonucleotides called 'chimeroplasts' or 'chimeraplasts' has been reported to be capable of inducing specific gene repair at remarkable frequency (104,108-111). Although the exact mechanism and the prerequisites imposed on the chimeric oligonucleotide structure remain poorly rationalised, the efficiency with which repair if single base pair mutations has reached up to 40% under some circumstances (109). There is still some controversy about the general applicability of the chimeraplasty, about its real efficiency and safety, and about the number of molecules that must accumulate into a cell to obtain satisfactory frequencies of repair. Also, for a certain time this technology could not be reproduced by research teams independent of the original discoverer. However, recent reports indicate that this technique may have finally worked in different laboratories (111). If indeed broadly applicable, chimeraplasty may become the method of choice for the treatment of many disorders caused by small genetic defects (single nucleotide mutations). The greatest advantage of this technique is that it promises to be exquisitely site-specific, thus to generate much less if at all undesired side-effects. if chimeraplasty can be ameliorated to achieve close to 100% repair, it may become a technique that reopens the option of germ line interventions, with all the bulky complement of ethical problems accompanying this dossier. In fact, taken at face value, chimeraplasty is the only available technology that fully deserves the denomination of 'gene therapy' in its strict sense.

2.5.2. Targeting

Owing to its principle, oligonucleotide-mediated therapy does not need to be strictly targeted at the level of delivery, since it is intrinsically aimed at precise interactions through base-pairing. However, given the very high costs of oligonucleotides, it will certainly be pharmacologically advantageous if the therapeutic oligos can be specifically delivered or accumulated to target tissues. We

envisage that our SMGD protocol (see 1.8) can be also adapted to ameliorate the accumulation of oligonucleotides in a tissue-preferential manner. Local delivery is so far the most popular option, although decoration with specific ligands for internalising receptors has been very promising, at least with liver-directed therapy with chimeraplasts (112).

2.5.3. Advantages / disadvantages

Compared to intact genes, oligonucleotides have molecular sizes of several orders of magnitude smaller (few thousands Daltons). This smaller size renders them more similar to conventional drugs, although they do not easily permeate through cell membranes. A second advantage is that oligonucleotides do not require bio-synthetic steps for their preparation, an thus can be more easily formulated under pathogen-free conditions.

The main disadvantage is that oligos tend to be degraded after a relatively short time, implying that the treatment of persisting diseases will require repeated administrations. The chimeraplasts are indeed able to produce a permanent effect but their current range of correction is limited to single point mutations.

2.5.4. Suitability and examples

In spite of the limitations mentioned above, oligonucleotides have been maintaining their therapeutic promises. After transfer of double stranded decoy oligonucleotides that titrate transcription factors controlling cell proliferation, V. Dzau and colleagues has been able to significantly reduce the incidence of intima-growth in vein transplants (39). In this case, the merit of the approach is that the transient treatment is sufficient to bring a permanent effect, since it sustains the non-degenerative adaptation of veins to the higher pressure. We have also already mentioned the spectacular correction rate obtained in rat models of a liver disorder (109) with chimeroplasty. Here also, a short treatment permits a long term therapeutic effect. Several antisense oligo approaches against protooncogenes are now in advanced

clinical testing. However, we have to wait these tests to assess the validity of this approach. Personally, we are quite sceptical about these anti-cancer therapies, specially since they don't offer a priori any bystander effect (see 1.2) and therefore seem less suitable for tumour eradication.

3. FINAL HURDLES AND CONCLUSIONS

3.1. Immune response and re-administration

This book is focussing on the molecular treatment of a disorder with auto immune and inflammatory components. In this kind of treatment it is absolutely imperative that the procedure should not imply reagents or manipulations that could unnecessarily activate the cellular or humoral immune system be it specific or innate. Therefore, the work with viral vectors of any kind should be considered with substantial caution. However, also non viral gene transfer could pose several problems when using entire genes, due to the innate reaction against unmethylated CpG-rich motifs. In general, re-administration is almost unavoidable with the current technology and this does not simplify the foreseeable clinical protocols for the treatment of chronic disorders with inflammatory components. Thus, until better control on the short-term and long-term immunogenicity of gene delivery systems can be obtained, gene therapy cannot be considered as a first priority for this class of disorders where it could ultimately exacerbate the outcome instead of bringing a therapeutical effect (113).

Finally, the immune system poses a problem also when the vectors themselves are clean of any pro-inflammatory properties. In fact, in some genetic loss-of-functions the resident gene is either deleted or totally non-functional. In this cases, the expression of the healthy gene product can lead to tissue rejection since it is detected as a 'foreign' antigen by the host immune system (23,69,114). For these cases, tolerisation strategies must be devised before considering gene transfer.

3.2. Safety considerations, from RCP to insertional mutagenesis

The immune reactions are not our sole hurdle in virally-assisted gene transfer. Some capsid proteins, while providing useful functions such as translocation and protection from degratative enzymes, are themselves toxic and can produce adverse reactions. In fact, most biologically assembled viral preparations contain a large excess (between 10 and 100 fold) of non functional viral particles. these particles can significantly contribute to the overall toxicity of the gene transfer procedure. Also we have mentioned that no biological recombinant viral preparation can be a priori guaranteed to be free of adventitious recombinants that have re-acquired viral genes sufficient for autonomous replication (the so-called RCPs, see 2.4.1). Even if the incidence of RCP can be reduced to less than one event in 10exp[14], this may pose serious constraints and cause severs costs augmentations to the industrial preparation of clinical materials.

The vectors that currently permit permanent transfer do not have ways of controlling the site of integration of the transgene. Thus, every cellular integration event is in principle an insertional mutagenesis event. At the somatic level, the large amount of insertional mutagenesis has the potential of generating pro-tumorigenic cells by activating protooncogenes. This relegates the use of integrating vectors for the treatment of life-threatening diseases, where the risk of generating a secondary tumor is still acceptable. The random mutagenesis generates another dilemma if the gene delivery vector transforms germ cells. In this case we would have a large number of aditional mutations that would be inherited to subsequent generations, and the potential benefit for the treated individual could become a strong disadvantage for his/her progeny. These dilemmas will be solved when we will be able to assemble vectors that can permanently deliver transgenes in specific chromosomal

locations. Considering the current pace of progress, this goal should not be so far.

3.3. Pulling it all together

If a pragmatic reader had the patience to read all the good and bad news about the existing and prospected vectors, he/she may ask: but after all, which vector/delivery is suitable for my goal?

In the Table 1 we summarise the suitability of the currently available gene transfer methods (columns) for different types of treatment. the number of '+' signs indicates qualitatively the suitability of a given combination. We hope that this synopsis may help in the choice of the most appropriate combination.

TABLE 1

Table 1 Qualitative assessment of suitability of delivery vehicles

Application	ADV	AAV	HSV	HIV	OLI	PEM	GUN	LIP
Vaccination /prevention	++	(+)	(+)	(+)	-	-	+++	(+)
Acute treatment	++	-	-	-	++	-	-	+
Chronic treatment	-	++	++	+++	(+)	-	-	+
In vivo local delivery	++	++	++	++	++	+	++	+
In vivo systemic delivery	+	+	+	+	+	n	n	+
Ex vivo delivery	++	+	+	+++	++	++	+	++
Single administration	+++	+++	+++	+++	+	+	+	+
Repeated administration	(+)	+	(+)	++	+++	+	+++	+++
Treat loss-of-function	+++	+++	+++	+++	+	++	+	++
Treat gain-of-function	(+)	+	+	+	+++	-	-	+
Gene correction	-	-	-	-	+++	-	-	-

Symbols: Adv, Adenovirus vectors; Aav, adeno-associated virus vectors; Hsv, Herpes virus vectors; Hiv, lentiviral vectors; Oli, oligonucleotides; Pem, pressure or electroporation mediated delivery; Gun, biolistic or macroinjection; Lip, lipoplexes or polyplexes. Symbols for suitability: -, not suitable; (+) questionable; +, hardly suitable; ++ , offers several applications; +++, excellent choice; n, does not apply. Most of the indicated degrees of suitability are justified in this chapter.

3.4. Outlook:
Will rudimentary vectorology
with all its troubles
survive the emerging challenge of
stem cell therapy?

Stem cell research has been booming in the last months. Primordial cells for almost all the tissues, including the CNS, have been characterised and the major hope is that the ex vivo cultivation of those may permit tissue regeneration for various treatments (115,116). The most spectacular observation is certainly that some stem cells seem to be able to trans-determination, that is to give rise to differentiated cells that are different than the donor tissue (97,116,117). Many of the claims and reports in this field are not yet even in the peer-reviewed literature but have been propagated through press releases or news-agencies despatches. According to them, in a foreseeable future, it should be possible to explant bone marrow cells and later reconstruct muscle, nerves, bones, epidermis and other types of tissues from this original population. The mechanisms that govern the maintenance of the pluripotency and the commitment towards one or another lineage are still obscure, but they are so intensively studied that we can anticipate major breakthroughs within the next few years.

3.4.1 The worst and best case scenario

Being able to culture stem cells without losing their pluripotency and to then determine their commitment would pave the way to autologous organ reconstruction that could cure an immensely large number of degenerative disorders. If the disorder has a genetic component, the corresponding correction could be easily achieved with conventional gene transfection ex-vivo and corresponding selection of pre-characterised recombinant cell clones. This would render obsolete most of the efforts to obtain high efficiency gene delivery vectors. Those latter would only be required for those cases where a cell therapy is not indicated, such as in acute treatments or corrections of gain-of-function disorders.

Thus, taken at face value, cell therapy has all the hallmarks to become a superior procedure for the treatment of chronic conditions. The worst that could happen is when patents and human ambitions would transform the noble tendency towards a better therapy into a ferocious battle between gene therapists and cell therapists for the best slices of the health market.

The balance of the odds for gene or cell therapy could change drastically if gene correction procedures such as chimeroplasty (see 2.5.1) would confirm their efficacy or if hybrid vectors (see 2.3.1), artificial viruses (see 2.1.2) or coherently integrating vectors (see 1.3, 2.3.3, 3.1) would make the long-sought brakthrough. If any of those tools would become generally applicable, then gene transfer in vivo would certainly remain competitive, since it implies lower costs, shorter intervention time and probably also lower invasivity than cell therapy.

3.4.2. A final homily for gene transfer

We have recapitulated the aims and efforts towards developing tools and methods for efficient gene transfer. When taken pessimistically, one could imagine that the few, but highly celebrated, therapeutic achievements are condemned to remain anecdotal and one wonders why should the scientists continue in this direction that has brought more frustrations than successes. From the point of view of fundamental research, the answer is refreshingly simple. While trying to solve the engineering problem of gene therapy, scientists have re-discovered and partially solved old neglected problems related to cell biology, virology, molecular transport and degradation, cell surface properties, etc. Furthermore, the pre-clinical efforts have produced vectors that are phenomenal tools for fundamental research. Gene transfer vectors are already considered for hit-and-run gene alteration procedures that will permit temporally and spatially controlled gene knock-out or knock-in in experimental animals, a situation which is laborious to achieve with conventional transgenesis. Furthermore, the gene transfer vectors open the way to the experimentation with primary cell cultures, that are notoriously refractory to biochemical gene transfer. This will permit the functional study of

genes under semi- or fully-physiological conditions and a better understanding of the intricate interactions between gene products. Therefore, gene therapy has brought an immense flood of novel knowledge that will substantially accelerate the overall progress in experimental life sciences.

From the clinical/pragmatic point of view, the heroic efforts towards gene therapy must be regarded as necessary steps that have broken the ice and paved the way towards more efficacious molecular therapies. We should not forget that any technological progress, from the aeroplane to the computer, has started with prototypes that seem almost ridiculous when compared with the today's opportunities. But without these glorious steps we would still be devoid of such marvellous achievements and would still be gasping intellectual conjectures about their feasibility instead of enjoying their concrete advantages. So, let's keep going and be proud thereof!

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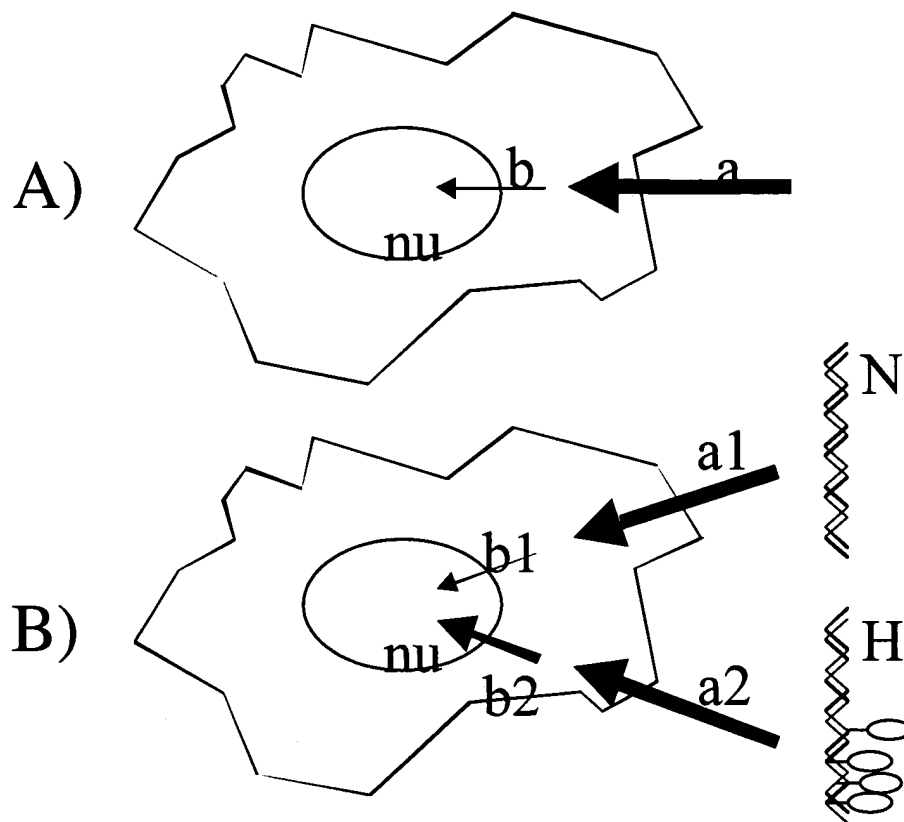
FIGURE 1, The two barriers for gene transfer and the principle of SMGD
A. In gene transfer the genetic material must pass two barriers: the cell membrane (a) and the nuclear envelope (b). Only a small proportion of the transferred nucleic acids undergoes nuclear transfer (compare thin arrow under b and thick arrow under a).
B. The concept of steroid mediated gene delivery. Conventional DNA (N), even if abundantly transfected (thick arrow marked a1) is only poorly translocated into the nucleus (thin arrow marked b1). Ligand-decorated DNA (marked H, where ovals with bar represent covalently linked ligands) is equally well transfected (a2) but better transported to the nucleus (b2) by the nuclear receptor that binds to the cognate ligand. This approach permits the selective facilitation of nuclear uptake of transgenes. In our laboratory we have proved this concept with model systems involving the glucocorticoid receptor (Ceppi et al. in preparation).
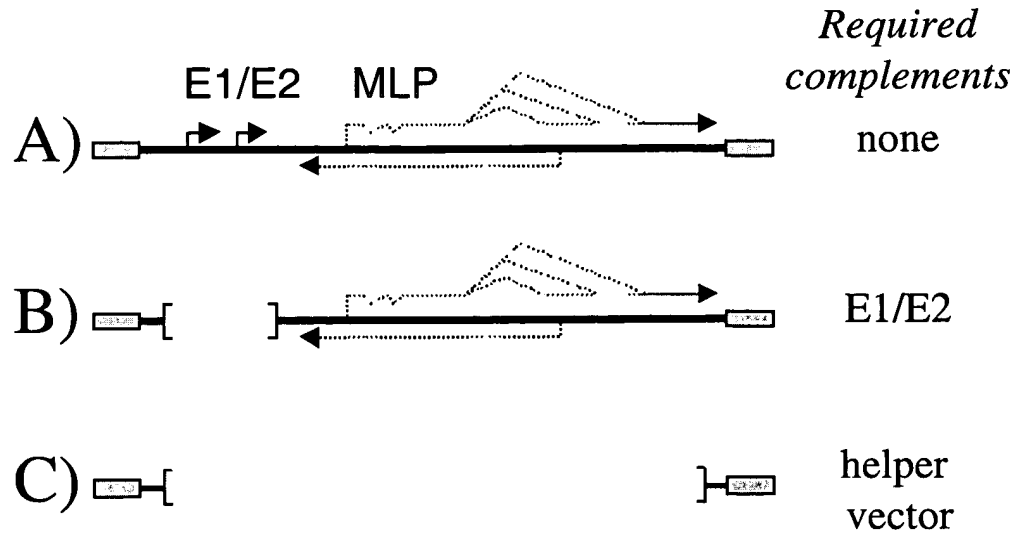
FIGURE 2: Genomes of wild type and recombinant adenovectors

A. The genome of wild type adenoviruses see text for references) is a linear 36 kb dsDNA flanked by inverted terminal repeats (hatched boxes) that are necessary for DNA replication and packaging. The indispensable early functions are E1 and E2 that are encoded by a 25% left portion of the genome (see E1 and E2). The late functions (capsid protein) are encoded by the remaining part and are mostly transcribed through the major late promoter (MLP) that gives rise to alternatively spliced mRNAs (broken dotted line), The late portion of the genome encodes in the opposite direction the viral DNA polymerase (dotted arrow pointing to left).

B. Replication-deficient adenovectors of the first generation are deleted in the early region and can be grown in packaging cells that provide the E1 and E2 functions. The deletion allows the accommodation of up to 8 kb of foreign DNA.

C. 'gutless' or 'high-capacity' adenovectors retain only the ITR region and can accommodate up to 32 kb of foreign DNA. These recombinant genomes can only be packaged in presence of a helper vector (see text).